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Functional signaling of membrane-bound TL1A induces IFN-γ expression

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ABSTRACT

TL1A, a TNF member implicated in autoimmune diseases, is a transmembrane protein that is processed to release soluble TL1A (TL1A-S). TL1A-S induces a Th1 response, although the functional significance of membrane-bound TL1A (TL1A-M) remains unknown. We generated TL1A-M expression in HEK-293 cells capable of binding DR3-Fc. Co-incubating IL-12/IL-18-primed $CD4^+$ T cells with HEK-293 cells expressing TL1A-M induced 3-fold increase in IFN-γ that was blocked by anti-TL1A Ab. These results demonstrate that TL1A-M can bind death domain receptor 3 (DR3) through cell–cell contact to induce downstream IFN-γ secretion enhancement. Anti-TL1A antibodies designed to treat immune diseases should be verified to block both endogenous TL1A forms.

1. Introduction

TL1A is a tumor necrosis factor (TNF) family member [1]. TL1A has been directly implicated in autoimmune disease pathogenesis such as rheumatoid arthritis [2–4], autoimmune encephalomyelitis [5,6], asthma [7] and inflammatory bowel disease [8,9]. TL1A was first reported to be expressed exclusively in human endothelial cells in response to TNFα or IL-1 [1]. Recent reports demonstrated inducible TL1A expression in macrophages [10,11], T cells [12,13], monocytes and dendritic cells [14–17]. TL1A is synthesized as a 28-kDa type II membrane protein with an extracellular domain that contains the TNF homology domain (THD) (Fig. 1A). THD can bind death domain receptor 3 (DR3), a TNF receptor (TNFR) family member, and induce signaling. TL1A can also bind to TR6/DcR3, a soluble decoy receptor that serves as negative regulator. Membrane-bound TL1A (TL1A-M) can be processed to release a 20-kDa soluble TL1A (TL1A-S) [1]. The identity of the protease responsible for TL1A processing is not yet known but possibly belongs to the matrix metalloproteinase family.

Although most TNF family ligands occur as both transmembrane and soluble forms, some are more functionally active in their membrane form (FASL) [18], some as soluble form (EDA [19,20] and APRIL) [21], and some in both forms (BAFF) [22]. TL1A-S has been shown to induce functional consequence when expressed by immune complex (IC)-induced monocytes [16] or when added in recombinant form [1,12,13]. However, it is not clear whether TL1A can act through its membrane-bound form via cell–cell contact. Recent studies suggest that expression of the two forms of TL1A is differentially regulated by the different cells. Monocytes and dendritic cells were shown to express both TL1A-M and TL1A-S in response to IC [15,16] or microbial organisms stimuli [17]. IC did not induce TL1A-S expression in lymphocytes [16]. Conversely, inducible expression of TL1A was observed only at the membrane level of T cells [12] but not in the cultured media (Gonsky and Deem, unpublished results). $CCR9^+CD4^+$ T cells were found to constitutively express TL1A-M [13]. In these reports, TL1A expression was shown to augment IL-12/IL-18-mediated IFN-γ production. However, it is not known if enhanced IFN-γ production could be attributed to TL1A-S or TL1A-M.

To address this question we engineered a construct expressing a non-cleavable TL1A-M and demonstrated that TL1A-M binds DR3 and enhances IFN-γ production in primed $CD4^+$ T cells. Our data imply that TL1A can function as membrane-bound protein and induce signaling through cell–cell contact.

2. Materials and methods

2.1. Preparation of Flag-tagged TL1A (A71D), TL1A (Δ66–75) and TL1A (Δ66–94)

To prepare full-length TL1A (TL1A-FL) expression vector, Flag-tagged upstream (pFlag-TL1A-FL), TL1A-FL sequence was PCR amplified from a pcDNA3.1-TL1A (Teva Pharmaceuticals) using the primers 5′-aaaaagcttatggccgaggatctgggactgag-3′ (forward) and 5′-aaaggatccctagagtaagaaggctccaaagaaggt-3′ (reverse). TL1A-S (aa61–251) from pCMV1-Flag-TL1A-S (Teva Pharmaceuticals) was removed following HindIII/BamHI digestion and replaced with

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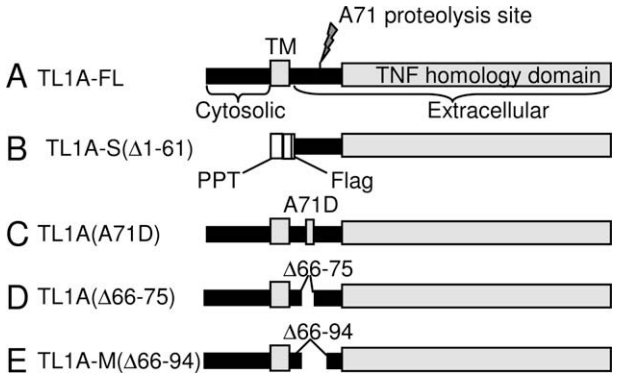

Fig. 1. Schematic diagram of modifications in TL1A. FL, full length; TM, transmembrane; TL1A-S, soluble form of TL1A; PPT, preprotrypsin; TL1A-M, non-cleavable form of TL1A.

TL1A-FL. The upstream preprotrypsin leader sequence was deleted from pFlag-TL1A-FL by site directed mutagenesis using the primers 5′-cgtcagaattaattcaccatg-3′ (forward) and 5′-gtcgtcatcgtctttgtagtc-3′ (reverse). To prepare TL1A-FL expression vector, Flag-tagged downstream (pTL1A-FL-Flag), TL1A-FL sequence was PCR amplified from pCDNA3.1-TL1A, using the primers 5′-aaagaattcatggccgaggatctgggactgag-3′ (forward) and 5′-aaactcgagtaagaaggctccaaagaaggt-3′ (reverse), and inserted into pIRES-hrGFP-1a (Stratagene Santa Clara, CA). TL1A(A71D), TL1A(Δ66–75) and TL1A(Δ66–94) expression vectors were generated by mutating pFlag-TL1A-FL and pTL1A-FL-Flag expression vectors using the primers 5′-gaggcctgtgtgcagttccaagatctaaaaggacagg-3′ (forward) and 5′-cctgtccttttagatcttggaactgcacacaggcctc-3′ (reverse) for Ala71 to Asp substitution, 5′-cccagggagaggccgagtttgcaccttc-3′ (forward) and 5′-gaaggtgcaaactcggcctctccctggg-3′ (reverse) for aa66–75 deletion and 5′-ccagggagaggccccaagggcacacc-3′ (forward) and 5′-ggtgtgcccttggggcctctccctgg-3′ (reverse) for aa66–94 deletion. All constructs were verified by digestion and sequencing.

2.2. Immunoblotting and DR3 binding assay

HEK-293 cells were obtained from and cultured as recommended by the American Tissue Culture Collection. Cells were transfected with TL1A-FL, TL1A-M, TL1A-S expression vectors or mock transfected with pIRES-hrGFP using effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Twenty-four hours post-transfection cells and supernatants were collected, and cells were lysed in 0.5 ml RIPA buffer (Cell Signaling, Beverly, MA). For DR3 binding assay cell lysates were incubated with 3 μg recombinant DR3-Fc (Alexis Biochemicals, San Diego, CA) at 4 °C for an hour followed by addition of 20 μl true-blot beads (eBioscience, San Diego, CA) and incubated at 4 °C with rotation, overnight. Beads were washed thoroughly and boiled in reducing sample buffer to elute precipitated proteins. Protein samples were separated on 10% or 15% SDS–PAGE and immunoblotted with monoclonal anti-Flag (Sigma, St. Louis, MO), or monoclonal anti- hTL1A Abs (04H08, 16H02) (Teva Pharmaceuticals) followed by IRDye 680 goat anti-mouse Ab (LI-COR Biosciences, Lincoln, NE). Membranes were imaged using the Odyssey infrared imaging system (LI-COR Biosciences).

2.3. Immunofluorescence

HEK-293 cells, plated on poly-D-lysine treated coverslips, were transfected with TL1A-FL, TL1A-M or TL1A-S expression vectors. Twenty-four hours post-transfection cells were fixed with 4% paraformaldehyde for 20 min. For Flag-tag staining, cells were gently permeabilized with 0.5% Tween-20 for 10 min. Fixed cells were incubated with humanized anti-TL1A (h1B4) (Teva Pharmaceuticals) or anti-Flag Abs overnight followed by incubation with PE-tagged Goat anti-human IgG or FITC-tagged Goat anti-mouse IgG (Jackson Laboratories Inc., Bar Harbor, ME) respectively for 1 h. Coverslips were mounted on glass-slides, using prolong gold mounting media (Invitrogen, Carlsbad, CA). Slides were imaged with wide-field Nikon microscope (Eclipse TE2000) equipped with an arc lamp, using Texas-red and FITC filter cubes with a 40× air emersion objective.

2.4. Flow cytometry

HEK-293 cells transfected with TL1A-FL, TL1A-M or TL1A-S expression vectors were incubated with humanized anti-TL1A (h1B4) or human IgG (Teva Biopharmaceuticals), washed and stained with FITC-tagged GαHu IgG. Cells were fixed with 1% paraformaldehyde and analyzed on a flow cytometer (FACScan).

2.5. $CD4^+$ T cells isolation and culture

Blood was obtained from normal donors after informed consent in accordance with procedures established by the Cedars-Sinai Institutional Review Board. PBMC were isolated as previously described [15]. $CD4^+$ T cells were isolated from PBMC using Human CD^+4 T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and incubated in RPMI complete medium.

2.6. Co-culture of $CD4^+$ T cells with transiently transfected 293 cells

Twenty-four hours after isolation, human IL-12 (final concentration, 2 ng/ml; PeproTech) and human IL-18 (final concentration 50 ng/ml; R&D systems, Minneapolis, MN) were added to $CD4^+$ T cells cultured in RPMI complete medium. For TL1A blocking Ab experiments, a primed $CD4^+$ T cells population was divided into two and blocking TL1A Ab (h1B4) or hIgG (control), was added at final concentration of 15 μg/ml. $CD4^+$ T cells were then added (5×10^5 cells/well) to HEK-293 cells (5×10^4 cells/well) plated in 24 well dishes that were transiently transfected with TL1A-FL, TL1A-M, TL1A-S expression vectors or mock transfected with pIRES-hrGFP 24 h earlier. Recombinant soluble TL1A (Teva Pharmaceuticals), at final concentrations of 2 ng/ml or 20 ng/ml and cells were co-cultured for 24 h.

2.7. Analysis of TL1A and IFN-γ by ELISA

TL1A was quantified in undiluted supernatants from transfected HEK-293 cells or from co-cultured experiments, using ELISA as described previously [15] with capture mAbs 04H08 or h1B4 and detector mAb biotinylated-16H02. IFN-γ was quantified by ELISA as previously reported [12].

3. Results

3.1. Generation and characterization of soluble and membrane forms of TL1A

Migone et al. [1] reported that the N-terminus of soluble TL1A, purified from 293T cells transfected with full-length TL1A, was Leu72. Based on this finding we designed TL1A-M expression DNA vectors that lack the putative proteinase cleavage site using several site-mutations/deletions around Leu72 in full-length TL1A encoding sequence (Fig. 1C–E). TL1A-FL and modified TL1A forms were Flag-tagged downstream (Fig. 2A) or upstream (Fig. 2B and C). TL1A-S (aa61–251) expression vector included an upstream Flag-tag and a preprotrypsin leader secretion sequence

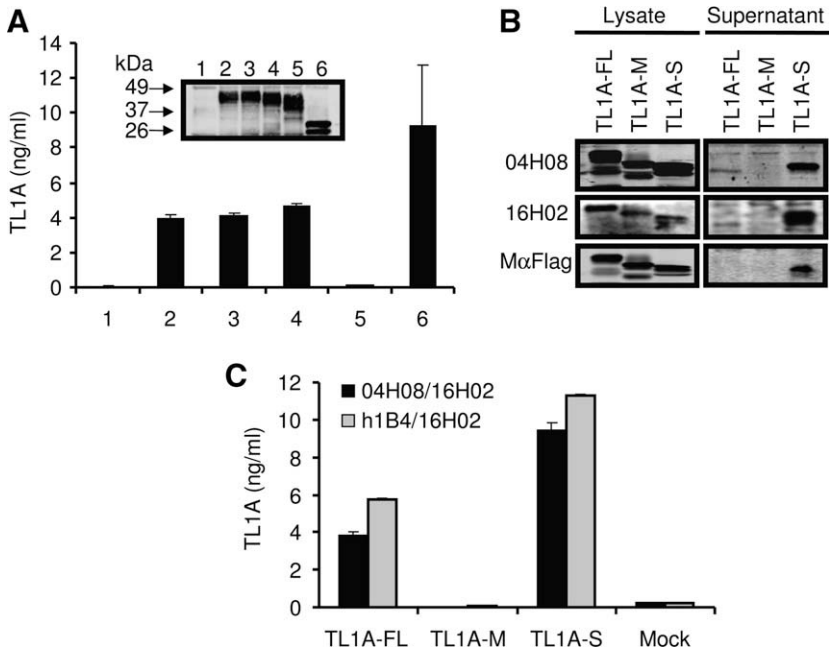

Fig. 2. Detection of TL1A expression constructs. (A) HEK-293 cells were transfected with downstream Flag-tagged TL1A expression vectors: (1) empty vector; (2) TL1A-FL; (3) A71D; (4) Δ66–75; (5) Δ66–94 and (6) upstream Flag-tagged TL1A-S. Supernatants were collected for ELISA measurements using anti-Flag coating and 16H02 detection Abs. Representative of two experiments with similar results. (Inset) Immunoblot of cell lysates using anti-Flag MAb. (B) Comparison of expression of upstream Flag-tagged TL1A variants in cell lysates (left panel) and supernatants (right panel). Immunoblots were analyzed with 04H08, 16H02 or anti-Flag Abs (C). Supernatants from (B) were measured by ELISA using 04H08 or h1B4 coating and 16H02 detection Abs. Representative of two experiments with similar results.

(Fig. 1B). To test for expression, we transfected HEK-293 cells with the TL1A expression vectors and collected cells and supernatants 24 h later. Immunoblot analysis of whole cell lysates displayed TL1A bands of the expected sizes (Fig. 2A, inset). ELISA measurements detected high levels of secreted or processed soluble TL1A in supernatants of HEK-293 cells transfected with TL1A-S or TL1A-FL expression vectors (Fig. 2A). Migone et al. [1] has previously shown that full-length TL1A, transfected in 293T cells, can be processed without addition of external proteinases. Out of the three mutated TL1A forms flanking Leu72, only aa66–94 deletion resulted in loss of detectable TL1A in the supernatant. Expression of non-cleavable TL1A-M was further confirmed by immunoblot comparing protein expression in the cell lysate to that within the supernatant (Fig. 2B). Two monoclonal anti-TL1A antibodies against distinct epitopes, 04H08 and 16H02, were utilized. Both MAb detected expression of full-length, membrane-bound and secreted forms of TL1A within the cell lysates. Supernatant from TL1A-FL expressing cells exhibited a weak but detectable lower molecular weight band indicating a cleaved soluble TL1A form (Fig. 2B). In contrast, no band was seen for TL1A-M. TL1A expression in the supernatants was also measured by ELISA using 04H08 or the humanized TL1A antibody h1B4 for capture and 16H02 for detection (Fig. 2C). h1B4 could not be used for immunoblotting as it does not recognize denatured TL1A (data not shown). Although expression of TL1A-FL and TL1A-S were detected by both antibody combinations, no combination of antibodies was able to detect secreted TL1A in the supernatant of cells transfected with TL1A-M.

The presence of TL1A-M at the plasma membrane was further verified by surface staining using immuno-histochemistry and FACS analysis (Fig. 3). As expected, TL1A-FL demonstrated location at the plasma membrane (Fig. 3A and B, top panels). Expression of TL1A-M (Fig. 3A and B, middle panels) was likewise detected by surface staining. In cells expressing TL1A-S, only dim vesicles were observed indicating the exocytosis process (Fig. 3A, lower left panel) which was likewise detected by FACS analysis as staining with weak florescent intensity (Fig. 3C). All three TL1A forms were shown to be expressed in cells when permeabilized and detected for Flag (Fig. 3A, right panel).

3.2. TL1A-M binds DR3

TL1A-FL and TL1A-S (aa72–251) were shown to bind DR3 as well as TR6 [1]. To verify that TL1A-M, expressed in HEK-293 cells, is capable of binding the receptor, TL1A-M, TL1A-FL and TL1A-S, expressed in HEK-293 cells, were incubated with recombinant Fc-tagged extracellular domain of DR3 (DR3-Fc). DR3-Fc was precipitated and samples were immunoblotted using anti-Flag Ab. As shown in Fig. 4, TL1A-M, like TL1A-S and TL1A-FL, bound to DR3-Fc, indicating that deletion of amino acids 66–94 did not disturb the binding between DR3 and TL1A-M.

3.3. TL1A-M enhances IFN-γ production by $CD4^+$ T cells

Meylan et al. [5] reported that DR3 is expressed primarily on T cells with the highest expression on $CD4^+$ T cells. In order to determine whether TL1A-M can activate DR3 and induce downstream signaling, we tested the functional effect of TL1A-M on $CD4^+$ T cells. We used an IL-12/L-18-primed $CD4^+$ T cell culture system previously described by our group [15,23] in which IFN-γ production by $CD4^+$ T cells is measured as an end point. TL1A has shown to potentiate secretion of IFN-γ in this system. The levels of secreted IFN-γ in response to TL1A-M were compared to those in response to TL1A-FL, TL1A-S and mock transfected cells. As shown in Fig. 5A we observed a statistically significant increase of 3-fold in IFN-γ production by IL-12/IL-18-primed $CD4^+$ T cells co-cultured with TL1A-M expressing HEK-293 cells compared with mock transfected cells (P value $\leqslant 0.04$). Moreover, IFN-γ enhancement in response to TL1A-M displayed 50% of the activity of that induced by TL1A-FL, which exhibits both soluble ad membrane-bound expression (Fig. 2). A further 2–5-fold enhancement was detected for TL1A-S, compared to TL1A-FL and TL1A-M respectively

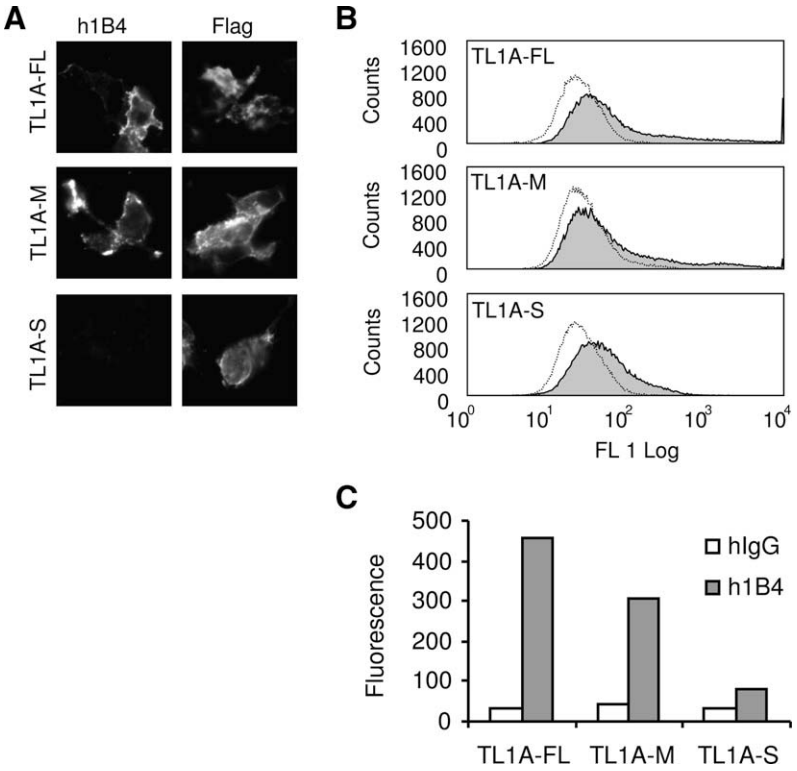

Fig. 3. Surface expression of TL1A constructs. (A) Representative Immunohistochemical images of expressed upstream Flag-tagged TL1A-FL, TL1A-M or TL1A-S. Surface TL1A was detected with h1B4 (no permeabilization). Total cell TL1A was with anti-Flag (after permeabilization). PE-tagged GαHu or FITC-tagged GαM IgG were used for visualization. (B) Flow cytometry plots of 293 cells expressing TL1A-FL, TL1A-M or TL1A-S. Surface TL1A was detected with h1B4 (complete line with gray background) or hIgG as negative control (dotted line no background). (C) Mean fluorescent intensities of flow cytometry data from (B).

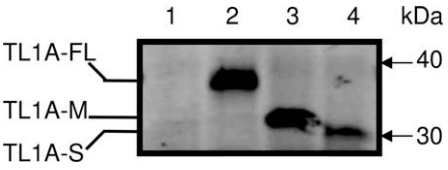

Fig. 4. Binding of the different TL1A forms to recombinant DR3-Fc. HEK-293 cells were transfected with upstream Flag-tagged TL1A-FL (2), TL1A-M (3) or TL1A-S (4) expressing vectors or with an empty vector (1). Cells were lysed and incubated with recombinant Fc-tagged extracellular domain of DR3 (rDR3-Fc) and TrueBlot beads. Proteins, released from the beads were loaded onto SDS-PAGE and analyzed using MAb against Flag.

(Fig. 5A and Table 1). To assess the magnitude of functionality of TL1A-M we compared the effect of TL1A-M to increasing amounts of recombinant TL1A added to mock transfected HEK-293 cells co-cultured with primed T cells (Fig. 5B). The magnitude of IFN-γ enhancement in response to TL1A-M was comparable to 2 ng/ml recombinant TL1A. To confirm that the enhancement in IFN-γ production was in response to TL1A, TL1A blocking Ab (h1B4) or control hIgG were added to the co-culture. Enhanced IFN-γ production was a result of TL1A signaling as TL1A blocking Ab inhibited the increase in IFN-γ (Fig. 5B black bars). These results indicate that membrane bound TL1A function similarly as soluble TL1A in induction of IFN-γ production by $CD4^+$ T cells.

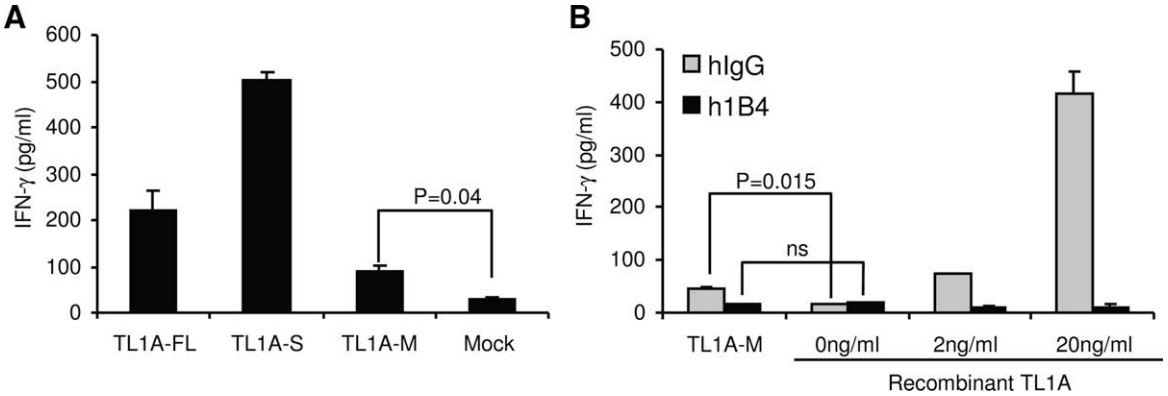

Fig. 5. TL1A-M functionally enhances IFN-γ secretion. (A) HEK-293 cells transfected with TL1A-FL, TL1A-S or TL1A-M expressing vectors or mock transfected with empty vector alone, were co-incubated for 24 h with IL-12/IL-18 primed $CD4^+$ T cells. Supernatants were collected and IFN-γ levels were measured by ELISA. Representative of three experiments with similar results. (B) Cells transfected with TL1A-M or mock transfected were co-incubated with primed-$CD4^+$ T cells as above in the presence of blocking TL1A Ab (h1B4) or hIgG isotype control. To functionally quantify the effects of TL1A-M, in parallel recombinant TL1A (aa72–251) was added to mock samples at final concentrations of 0, 2 or 20 ng/ml and IFN-γ levels were measured using ELISA. Representative of three experiments with similar results.

Table 1
Results summary.

	Cell surface	Soluble	Functional
TL1A-FL	+	+	++
TL1A-S	–	+	+++
TL1A-M	+	–	+

4. Discussion

Although studied intensively in the recent years, most of our knowledge about TL1A structure and function has been established mainly through its soluble form. Since it is not yet known what proteinase cleaves membrane TL1A into its soluble form, TL1A-S effect could not be eliminated, to enable investigating the potential effect of membrane-bound TL1A.

In this study we created a membrane-bound TL1A expressing vector by removing aa66–94 from full-length TL1A (Figs. 2 and 3). Interestingly, out of the three modifications done around the potential cleavage site, only TL1A (Δ66–94) generated a non-cleavable membrane-bound product. Single mutation at Ala71, as well as deletion of aa66–75, did not prevent TL1A cleavage. These results might suggest a proteinase with wide substrate recognition or the possibility of another cleavage site close by, other then between Ala71 and Leu72.

We further demonstrated that TL1A-M was capable of binding DR3 (Fig. 4) and inducing immune response through $CD4^+$ T cells (Fig. 4). We observed a statistically significant 3-fold increase of IFN-γ production in response to TL1A-M compared to mock, which was approximately 50% of that observed in response to TL1A-FL. This increase is similar to our observation in earlier studies with IL-12/IL-18-primed $CCR9^+$ $CD4^+$ T cells [13]. A further enhancement of IFN-γ production is induced by TL1A-S. The results are summarized in Table 1. The difference in IFN-γ enhancement between the different TL1A forms was expected, since the current setup is not optimal for cell–cell contact-mediated signaling required for membrane-bound TL1A. There are several limitations in this setup: (1) only a fraction of transfected HEK-293 cells express the transcript, (2) only a subset of $CD4^+$T cells population responds to TL1A, and (3) 293 cells are adherent cells thus the effect of TL1A-M, which occur thorough cell–cell contact, is spatially limited to a monolayer. However, we did observe that reducing the reaction volume, as well as increasing $CD4^+$ T cells concentration, did increase TL1A-M effect (data not shown). Despite these limitations the enhancement in IFN-γ was confirmed to be in response to TL1A presence, since TL1A blocking Ab inhibited IFN-γ increase. The effect of TL1A-M on IFN-γ enhancement was comparable to 2 ng/ml recombinant TL1A, which is within the range of TL1A expression observed in supernatants of IC-stimulated monocytes [15].

In earlier studies we observed differential TL1A expression between the different immune cells. APC demonstrated both membrane-bound and soluble TL1A when induced by IC [15] or by microbial organisms [17]. However, TLIA expression in T cells, was found only at the membrane and it has been shown to function in increased IL-12/IL-18 induced IFN-γ production in $CCR9^+$ $CD4^+$ cells [12,13]. This suggests that T cells expressing membrane-bound TL1A come in contact with T cells expressing membrane DR3 to induce increased IFN-γ production. The results in this study demonstrate the likelihood of this scenario.

Studies in mouse models, using neutralizing TL1A antibody, have shown to significantly attenuate chronic colitis [8] and protect against systemic rheumatoid arthritis [2]. In these studies TL1A antibody was designed to recognize and block soluble recombinant TL1A. Only a partial recovery of inflamed tissue in response to TL1A antibody was observed in both studies. Given that both membrane and soluble TL1A are functionally competent, antibodies designed to block TL1A function to prevent/treat immune mediated diseases, should be verified to recognize and block both forms of endogenous TL1A functions, not just the recombinant form.

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